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Sulfate-reducing bacterial community response to carbon source amendments in contaminated aquifer microcosms

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Abstract

Microbial sulfate reduction is an important metabolic activity in many reduced habitats. However, little is known about the sulfate-reducing communities inhabiting petroleum hydrocarbon (PHC)-contaminated freshwater aquifer sediments. The purpose of this study was to identify the groups of sulfate-reducing bacteria (SRB) selectively stimulated when sediment from a PHC-contaminated freshwater aquifer was incubated in sulfate-reducing aquifer microcosms that were amended with specific carbon sources (acetate, butyrate, propionate, lactate, and citrate). After 2 months of incubation, the SRB community was characterized using phospholipid fatty acid (PLFA) analysis combined with multivariate statistics as well as fluorescence in situ hybridization (FISH). Molybdate was used to specifically inhibit SRB in separate microcosms to investigate the contribution of non-SRB to carbon source degradation. Results indicated that sulfate reduction in the original sediment was an important process but was limited by the availability of sulfate. Substantially lower amounts of acetate and butyrate were degraded in molybdate treatments as compared to treatments without molybdate, suggesting that SRB were the major bacterial group responsible for carbon source turnover in microcosms. All of the added carbon sources induced changes in the SRB community structure. Members of the genus *Desulfobulbus* were present but not active in the original sediment but an increase of the fatty acids 15:1ω6c and 17:1ω6c and FISH results showed an enrichment of these bacteria in microcosms amended with propionate or lactate. The appearance of cy17:0 revealed that bacteria affiliated with the *Desulfobacteriaceae* were responsible for acetate degradation. *Desulfovibrio* and *Desulfotomaculum* spp. were not important populations within the SRB community in microcosms because they did not proliferate on carbon sources usually favored by these organisms. Metabolic, PLFA, and FISH results provided information on the SRB community in a PHC-contaminated freshwater environment, which exhibited stimulation patterns similar to other (e.g. marine) environments.

Keywords: Sulfate-reducing bacterium; Petroleum hydrocarbon contaminated aquifer; Phospholipid fatty acid; Fluorescence in situ hybridization; Low molecular mass organic acid; Microcosm

1. Introduction

Dissimilatory microbial sulfate reduction is an important metabolic activity in many reduced environments such as marine sediments [1], anoxic groundwater and soil [2], anaerobic sludge [3,4], and contaminated aquifers [5]. This activity is mediated by the metabolically diverse group of sulfate-reducing bacteria (SRB) [6–8]. Certain SRB are known to utilize environmental contaminants such as petroleum hydrocarbon (PHC) constituents (e.g. benzene, toluene, ethylbenzene, xylenes, polycyclic aromatic hydrocarbons, alkanes) or halogenated compounds directly as a source of carbon and energy [9,10]. In addition, low molecular mass organic acids such as acetate, propionate and butyrate are common metabolic intermediates in the degradation of PHC [11] and may also serve as carbon sources for SRB in contaminated environments [12]. Acetate, propionate and butyrate are the principal organic acids in many anaerobic ecosystems [13,14]. In marine sediments, organic acids derived mostly from fermentation processes serve as the SRB's main carbon source [14,15] and lactate was identified to be an important carbon source for sulfate reduction in freshwater sedi-

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ments [16,17]. However, little is known about the role that such low molecular mass organic acids may play in the community pathway of PHC degradation in contaminated aquifers.

In such aquifers, biogeochemical processes are intimately linked with the types of microorganisms present. Hence, information on identity and function of bacteria is essential to better understand these processes. Direct information on the identity of SRB communities may be obtained using laboratory molecular methods such as fluorescence in situ hybridization (FISH) [18] or the analysis of phospholipid fatty acids (PLFA) [19]. A large database of PLFA profiles was developed from pure culture studies [3,20–26]. This database was successfully used to relate the presence or absence of specific PLFA to SRB community structure and dynamics in marine and brackish sediments and anaerobic sludge [3,27–29]. However, we must be aware that yet undiscovered SRB species and genera may inhabit anaerobic environments [30,31]. Only a few authors have characterized SRB communities in freshwater [32,33] and direct information on SRB in PHC-contaminated freshwater environments is even more limited [29].

The purpose of this research was to characterize the SRB populations selectively stimulated when sediment from a PHC-contaminated freshwater aquifer was incubated in sulfate-reducing aquifer microcosms that were amended with specific carbon sources (acetate, butyrate, propionate, lactate, and citrate). These microcosms were incubated for 2 months and amended carbon source and sulfate concentrations were periodically monitored. In separate microcosms, the SRB were inhibited by molybdate treatment to investigate the contribution of other microorganisms to carbon source degradation [34]. Microbial community structure in the original sediment and the sulfate-reducing microcosms was investigated by FISH and PLFA analysis combined with multivariate statistical analysis. Metabolic, PLFA, and FISH data provided a more comprehensive picture of the SRB community in a sulfate-reducing, PHC-contaminated freshwater aquifer.

2. Materials and methods

2.1. Sediment collection

Sediment for this study was collected from the bottom of a monitoring well in a PHC-contaminated aquifer in Studen, Switzerland (well S6) [35]. Groundwater at this well was anoxic (3 μM oxygen), nitrate concentration was below the detection limit (<1 μM) and sulfate concentration was 156 μM [36]. The sediment in this well contained high amounts of PHC (11 500 mg kg^{-1}, determined by infra-red spectrometry), which represented approximately 30% of the total organic carbon [36]. Sediment was recovered from the bottom of this well using a Peterson grab sampler and immediately transferred to 1-l glass bottles, which were constantly flushed with N_2 gas to maintain anaerobic conditions. The bottles were closed with butyl rubber stoppers and kept on ice during transport to the laboratory.

2.2. Microcosm experiments

For the construction of microcosms, 30 g of sediment was transferred to 117-ml serum bottles under N_2 atmosphere in an anaerobic glove box. Serum bottles were then closed using butyl rubber stoppers. Anoxic medium was prepared and dispensed into serum bottles to obtain a total volume of ~100 ml sediment/medium mixture according to [37]; then bottles were crimp-sealed. A freshly prepared, anoxic, sterile-filtered $FeSO_4$ solution was added to each serum bottle to achieve a final concentration of 3 mM sulfate. Thereafter, microcosms were amended with sterile, anoxic solutions that contained either acetate, propionate, butyrate, lactate or citrate to achieve a final concentration of 3 mM. Additional microcosm series were used as controls: 3 mM sulfate but not carbon source-amended (to account for consumption of endogenous carbon sources), autoclaved (to account for non-enzymatic processes, losses through sorption, etc.), and 3 mM molybdate-treated (to account for non-SRB activity) microcosms were inoculated with sediment samples and incubated in parallel with the above-described treatments. All treatments were performed in duplicates and incubated statically at 25°C in the dark.

In all microcosms, sulfate and carbon source concentrations were periodically (every 7–10 days) measured over a 2-month period. When concentrations of sulfate or carbon source were below ~0.5 mM, microcosms were replenished with sulfate or carbon source to achieve 3 mM final concentrations. Citrate was only once amended to microcosms because its concentration could not be monitored by the employed analytical method. After a 50-day incubation period, microcosms were sacrificed and 1-ml (total cell counts and FISH) and 20-ml (PLFA) aliquots of sediment sludge were used for community analyses.

2.3. Analytical methods

Concentrations of sulfate and amended carbon sources in the microcosms were quantified using a DX-100 ion chromatograph system (Dionex, Sunnyvale, CA, USA). For sulfate detection, the eluent was a bicarbonate buffer (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$). For carbon source (acetate, propionate, butyrate, lactate) measurements, a 5 mM $Na_2B_4O_7$ solution served as eluent. Using this method, the carbon sources all possessed the same retention time and could therefore not be distinguished from each other. Thus, while overall carbon source mineralization (oxidation to CO_2 and water) could be quantified, potential intermediates (e.g. acetate from incomplete

Table 1
Total consumed sulfate and mineralized carbon source (means ± range of duplicate microcosms), observed stoichiometric ratio, and theoretical stoichiometric ratios for complete and incomplete carbon source degradation in sulfate-reducing aquifer microcosms over a 50-day period

Microcosm treatment	Measured data			Theoretical data	
	Total SO_4^{2-} consumed (mmol)	Total carbon source mineralized (mmol)	Observed stoichiometric ratios ((mmol substrate) (mmol SO_4^{2-})$^{-1}$)	Theoretical stoichiometric ratios[a] ((mmol substrate) (mmol SO_4^{2-})$^{-1}$)	
				Complete	Incomplete
Acetate	1.19 ± 0.01	1.38 ± 0.00	1.2	1.00	–
Propionate	1.66 ± 0.37	1.27 ± 0.30	0.8	0.60	1.3
Butyrate	1.80 ± 0.15	0.80 ± 0.00	0.4	0.40	0.7
Lactate	1.80 ± 0.00	1.45 ± 0.00	0.8	0.66	2.0
Citrate	1.29 ± 0.14	n.d.[b]	n.d.	0.44	4.0
Sulfate only	1.08 ± 0.01	–	–	–	–
Acetate–molybdate	0	0.55 ± 0.01	–	–	–
Butyrate–molybdate	0	0.03 ± 0.03	–	–	–
Sterile acetate	0	0	–	–	–

[a]Based on Eqs. 1–5.
[b]n.d., not determined.

oxidation of propionate) could not be discerned. Methane was measured but not quantified in the headspace of all microcosms using the headspace technique as described in Bolliger et al. [35].

2.4. Total cell counts and in situ hybridization

Total cell counts and FISH analysis were performed only for one of each duplicate microcosm. Total cell counts were performed using 4′,6-diamidino-2′-phenylindole (DAPI) staining [38]. For FISH, we used the Cy3-labeled 16S rRNA oligonucleotide probes EUB338 targeting *Bacteria* [39], Arch915 for *Archaea* [40], SRB385 for δ-*Proteobacteria* including SRB [41], SRB385-Db for *Desulfobacteriaceae* [8], DSV698, DSV1292, DSD131, DSV214, and DSV407 for *Desulfovibrio* spp., DSB985 for *Desulfobacter*-like bacteria, probe 660 for *Desulfobulbus* spp. [4], and S-G-Dtm-0229-a-A-18 for *Desulfotomaculum* spp. [42]. The sediment sludge samples (1 ml) were fixed with paraformaldehyde or ethanol and stored according to Zarda et al. [38]. Except for probe S-G-Dtm-0229-a-A-18, for which the ethanol-fixed samples were used, paraformaldehyde-fixed samples were counted with all probes. Hybridizations with oligonucleotide probes as well as DAPI staining were performed under standard conditions [38] in the presence of formamide (10% for DSV214; 15% for S-G-Dtm-0229-a-A-18; 20% for Arch915, SRB385, SRB385-Db, DSB985 and DSD131; 30% for EUB338; 35% for DSV698 and DSV1292; 50% for DSV407; and 60% for probe 660). After hybridization and washing (NaCl concentrations in the wash buffer were 500 mM for DSV214; 318 mM for S-G-Dtm-0229-a-A-18; 250 mM for Arch915, SRB385, SRB385-Db, DSB985 and DSD131; 112 mM for EUB338; 88 mM for DSV698 and DSV1292; 31.2 mM for DSV407; and 15.6 mM for probe 660), slides were mounted with Citifluor solution (Citifluor AF2, Citifluor Ltd., London, UK). Visually detectable cells were counted according to Zarda et al. [38]. Detection limit of hybridized cells was 1% of total (DAPI-stained) cells.

Table 2
Total (DAPI-stained) cells and fluorescence in situ hybridization counts (% of DAPI-stained cells; values given as average ± standard deviation, $n = 4$) in the original sediment (S6) and microcosms with different treatments

Microcosm treatment	Total numbers of DAPI-stained cells (10^9 g^{-1} dry sediment)	Probe (target) (% of DAPI-stained cells)				
		EUB338 (*Bacteria*)	Arch915 (*Archaea*)	SRB385 (δ-*Proteobacteria*)	SRB385-Db (*Desulfobacteriaceae*)	660 (*Desulfobulbus*)
S6	1.3 ± 0.2	7.60 ± 4.3	1.3 ± 1.5	< 1	1.90 ± 0.6	< 1
Acetate	2.8 ± 0.4	33.2 ± 4.3	4.4 ± 1.1	9.80 ± 4.0	15.3 ± 1.2	< 1
Propionate	3.3 ± 0.6	36.7 ± 9.6	2.7 ± 2.4	15.1 ± 4.3	17.6 ± 3.7	4.3 ± 1.4
Butyrate	2.8 ± 0.6	42.4 ± 11	3.6 ± 1.6	11.5 ± 3.9	17.8 ± 5.1	< 1
Lactate	4.1 ± 1.0	31.8 ± 2.2	3.9 ± 2.2	11.4 ± 2.6	20.2 ± 1.6	5.4 ± 2.2
Citrate	3.8 ± 0.3	15.8 ± 2.9	1.2 ± 0.6	1.70 ± 0.7	10.3 ± 4.0	< 1
Sulfate only	2.1 ± 0.5	35.3 ± 13	< 1	6.90 ± 5.2	11.2 ± 5.5	2.1 ± 0.9
Acetate–molybdate	1.9 ± 0.5	6.10 ± 3.7	< 1	< 1	2.80 ± 0.6	< 1
Butyrate–molybdate	1.3 ± 0.1	6.60 ± 3.6	< 1	< 1	1.10 ± 1.4	< 1

Cells counted with probes DSV698+DSV1292, DSD131, DSV214, DSV407 (*Desulfovibrio*), DSB985 (*Desulfobacter*) and S-G-Dtm-0229-a-A-18 (*Desulfotomaculum*) were below the detection limit (< 1%).

2.5. Extraction and analysis of PLFA

PLFA were extracted by a modified Bligh–Dyer method [43] as described previously [44]. The PLFA were methylated according to standard protocol [44]. Gas chromatography (Hewlett Packard HP 5890 series II equipped with a HP Ultra 2 capillary column and a flame ionization detector) was used to separate the PLFA methyl esters. Identification of PLFA was carried out using the MIDI system (MIDI Inc. version 4.0). Each sample was analyzed in duplicate.

PLFA nomenclature is in the form of $A{:}B\omega C$, where A designates the total number of carbons, B the number of double bonds, and C the distance of the closest double bond from the aliphatic end of the molecule. The suffixes '-c' for *cis* and '-t' for *trans* refer to geometric isomers. The prefixes 'i-' and 'a-' refer to iso- and anteisomethyl branching.

In order to detect differences in PLFA profiles between microcosms and the original sediment, centered principal component analysis (PCA) was applied to the data set [45]. This multivariate statistical method reduces large data sets by forming uncorrelated linear combinations of the observed variables (in our case, PLFA) resulting in several principal components (PC) which progressively explain less of the variability (variance) of the data [46]. Differences of PLFA abundance between microcosms were tested for their statistical significance using analysis of variance (ANOVA), which is a method of testing differences between group means by comparing sample variances.

2.6. Calculations of stoichiometric ratios

Theoretical stoichiometric ratios (mol carbon source per mol sulfate) for incomplete carbon source degradation to acetate were obtained from the following reactions:

$$\text{Propionate}: \ \tfrac{4}{3}CH_3CH_2COO^- + SO_4^{2-} \rightarrow \tfrac{4}{3}CH_3COO^- + \tfrac{4}{3}HCO_3^- + HS^- + \tfrac{1}{3}H^+ \quad (1)$$

$$\text{Butyrate}: \ \tfrac{2}{3}CH_3(CH_2)_2COO^- + SO_4^{2-} \rightarrow \tfrac{2}{3}CH_3COO^- + \tfrac{2}{3}HCO_3^- + HS^- + \tfrac{1}{3}H^+ \quad (2)$$

$$\text{Lactate}: \ 2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+ \quad (3)$$

$$\text{Citrate}: \ 4COO^-CH_2COHCOO^-CH_2COO^- + SO_4^{2-} + 8H_2O \rightarrow 8CH_3COO^- + 8HCO_3^- + HS^- + 3H^+ \quad (4)$$

The theoretical stoichiometric ratio for acetate degradation was obtained from:

$$\text{Acetate}: \ CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (5)$$

Furthermore, we obtained theoretical stoichiometric ratios for complete carbon source degradation of propionate, butyrate, lactate, and citrate from combining Eqs. 1–4 individually with Eq. 5. Conversely, observed stoichiometric ratios were calculated as the ratio of the total amounts of sulfate consumed to carbon sources mineralized during the incubation period. In these calculations we assumed that added sulfate was used exclusively for the degradation of added carbon sources and that carbon sources were mineralized using sulfate as sole electron acceptor. Assimilation of carbon and sulfur by SRB was not taken into account in these calculations because it was assumed to be low [6].

3. Results

3.1. Sulfate and carbon source consumption

Sulfate was consumed over the 50-day period in all except the molybdate treatments and the autoclaved microcosm series (Table 1). Total sulfate consumption was highest in butyrate-, lactate-, and propionate-amended microcosms (1.66–1.80 mmol) and lowest in those microcosms without carbon source amendment (1.08 mmol). The amended carbon sources were not mineralized in the autoclaved microcosms and low mineralization occurred in the butyrate–molybdate treatment (0.03 mmol butyrate). Note, however, that some butyrate fermentation to acetate may have occurred. This process would have been undetected due to the employed analytical method. A considerable amount of acetate was mineralized in the acetate molybdate-treated microcosms (0.55 mmol). However, much higher carbon source mineralization was observed in acetate-, propionate-, butyrate- and lactate-amended microcosms. For these four treatments, observed stoichiometric ratios were closer to the theoretical (reaction equation-derived) values for complete than for incomplete carbon source oxidation (Table 1). Methane was detected in the headspaces of all microcosms except the autoclaved series (data not shown).

3.2. Total cell counts and in situ hybridization

Average total cell counts (DAPI) were highest in lactate-, citrate- and propionate-amended microcosms ($3.26–4.11\times10^9$ g^{-1} dry sediment), while the original sediment and the molybdate-treated microcosms showed lowest cell numbers ($1.28–1.94\times10^9$ g^{-1}) (Table 2).

Numbers of cells hybridizing with the general *Bacteria*-specific probe (EUB338) were low in molybdate-treated microcosms and in the original sediment (6.1–7.6% of total DAPI-stained cells) and approximately five times higher in acetate, propionate, lactate, and butyrate amendments, and in microcosms without extra carbon source amendment (31.8–42.4%). A considerable abundance of *Archaea*

(probe Arch915) was detected in acetate-amended microcosms (4.4%), in contrast to microcosms treated with molybdate and without extra carbon source amendment (< 1%). Cell counts with probes SRB385-Db and SRB385 followed approximately the same trend as results of probe EUB338. Counts were always higher with probe SRB385-Db than with SRB385. With these probes, we determined highest cell numbers in propionate-, lactate- or butyrate-amended microcosms (SRB385-Db and SRB385, 17.6–20.2 and 11.4–15.1%, respectively) and lowest cell numbers in the original sediment and in molybdate-treated microcosms (< 1–2.8%). The sum of cells hybridizing with both general SRB probes (SRB385 plus SRB385-Db) relative to cells hybridizing with probe EUB338 ranged from 29% (molybdate–butyrate treatment) to 99% (lactate amendment). The genus *Desulfobulbus* (probe 660) was detected in several microcosms in significant amounts (propionate and lactate, 4.3 and 5.4%, respectively). Conversely, in all aquifer microcosms, cells belonging to specific subgroups within the *Desulfobacteriaceae* (probe DSB985), *Desulfovibrionaceae* (probes DSV1292, DSV698, DSD131, DSV214, and DSV407), and *Desulfotomaculum* (S-G-Dtm-0229-a-A-18) were below the detection limit (< 1%).

3.3. Phospholipid fatty acids

Using the MIDI system, 91 ± 6% of the detected compounds were identified as PLFA. The PLFA profiles in microcosms were dominated by even *C*-numbered PLFA (80% of total PLFA), while on average 20% of total PLFA were branched and 54% were monounsaturated. The most abundant compounds were 18:0 (21%), 18:1ω7c (9%), 18:1ω9c (9%), and 16:0 (9%). In the original sediment, 10me16:0 and 17:1ω6c were present while cy17:0, 15:1ω6c, and i17:1ω7c were lacking.

Principal component analysis of PLFA profiles using all of the detected 69 compounds did not result in a reasonable separation of microcosms into groups (not shown). Therefore, a second PCA was conducted using 16 PLFA (Table 3), which are known to be present in SRB [3,20–26], plus the ubiquitous 16:0. Figs. 1 and 2 show the first two components of this PCA represented by the two axes, which together explained 69% of the variance of the PLFA data. Microcosms that group closely together in Fig. 1 possess similar PLFA compositions. Based on Fig. 1, we distinguished three groups of microcosms: the first group contained the original sediment, the molybdate treatments and microcosms without carbon source amendments, the second the lactate and propionate microcosms and the third the acetate, butyrate, and citrate microcosms.

The factorial map in Fig. 2 shows the ordination scores of the PLFA. The compounds 17:1ω6c, 15:1ω6c, and 15:0 on the one hand and cy17:0 and 10me17:0 on the other hand separated along the first axis. In addition, these two PLFA groups separated from all other PLFA along both axes. Those PLFA in the factorial map in Fig. 2 that lie close to microcosms in Fig. 1 when the plot origins are superimposed are likely to have a high relative abundance in those microcosms. Hence, cy17:0 and 10me17:0 were

Table 3
Relative abundance of SRB-related PLFA (% relative to the total, 17 of a total of 69 PLFA are shown) detected in aquifer microcosms (values ± standard deviations of four measurements (two in each of the duplicate microcosms))

Microcosm treatment	PLFA								
	i15:0	a15:0	15:1ω6c	15:0	16:1ω7c	16:0	10me16:0	a17:1	a17:1ω9c
S6	2.3 ± 0.3	1.8 ± 0.2	b.d.	1.5 ± 0.2^{b}	8.5 ± 0.4	7.70 ± 0.1	3.1 ± 0.2^{a}	1.2 ± 0.1	b.d.
Acetate	1.5 ± 0.2	1.1 ± 0.1	b.d.	1.6 ± 0.2^{b}	5.5 ± 1.2	10.0 ± 0.3	2.8 ± 0.8^{a}	1.0 ± 0.1	1.0 ± 0.3
Propionate	1.6 ± 0.5	1.0 ± 0.3	0.4 ± 0.3^{a}	2.3 ± 0.8^{a}	5.6 ± 1.9	8.20 ± 1.3	1.8 ± 0.5^{b}	1.5 ± 0.8	0.6 ± 0.5
Butyrate	1.3 ± 0.2	1.0 ± 0.2	b.d.	1.4 ± 0.2^{b}	7.7 ± 0.9	10.8 ± 0.6	2.4 ± 0.4^{a}	1.1 ± 0.1	0.8 ± 0.1
Lactate	1.7 ± 0.4	1.2 ± 0.3	0.3 ± 0.3^{a}	2.7 ± 0.7^{a}	5.1 ± 0.9	8.40 ± 0.9	2.1 ± 0.7^{b}	1.1 ± 0.6	0.9 ± 0.2
Citrate	1.4 ± 0.2	1.1 ± 0.2	b.d.	1.4 ± 0.4^{b}	6.0 ± 1.4	10.1 ± 0.3	2.1 ± 0.6^{b}	1.1 ± 0.4	0.7 ± 0.2
Sulfate only	1.4 ± 0.4	1.1 ± 0.4	b.d.	1.3 ± 0.6^{b}	7.8 ± 3.9	8.30 ± 0.8	2.1 ± 0.4^{b}	1.3 ± 0.3	0.9 ± 0.3
Acetate–molybdate	1.6 ± 0.3	1.2 ± 0.2	b.d.	0.4 ± 0.5^{c}	6.0 ± 1.2	9.00 ± 0.8	2.4 ± 0.6^{a}	2.5 ± 0.6	1.3 ± 0.4
Butyrate–molybdate	1.0 ± 0.2	0.9 ± 0.1	b.d.	1.3 ± 0.3^{b}	5.4 ± 1.9	9.00 ± 0.7	1.7 ± 0.4^{b}	1.4 ± 0.4	0.8 ± 0.2
	i17:0	17:1ω8c	17:1ω6c	cy17:0	10me17:0	18:1ω7c	10me18:0	cy19:0	
S6	1.7 ± 0.1	1.5 ± 0.2^{c}	2.1 ± 0.2^{b}	b.d.	0.5 ± 0.3	15.1 ± 0.9	2.4 ± 0.6	1.7 ± 1.0	
Acetate	1.3 ± 0.3	1.0 ± 0.2^{bc}	b.d.	2.3 ± 0.6^{b}	0.4 ± 0.2	8.8 ± 1.1	1.1 ± 0.2	0.8 ± 0.1	
Propionate	1.2 ± 0.4	1.1 ± 0.4^{bc}	5.4 ± 2.0^{a}	b.d.	0.3 ± 0.3	7.5 ± 3.5	1.3 ± 0.5	0.4 ± 0.4	
Butyrate	1.4 ± 0.3	1.2 ± 0.3^{bc}	b.d.	2.9 ± 0.2^{c}	0.6 ± 0.2	9.7 ± 1.6	1.8 ± 0.3	0.9 ± 0.1	
Lactate	1.5 ± 0.1	1.4 ± 0.6^{bc}	4.0 ± 1.9^{a}	b.d.	0.3 ± 0.2	7.5 ± 2.8	1.1 ± 0.6	0.8 ± 0.4	
Citrate	1.0 ± 0.3	1.1 ± 0.3^{bc}	b.d.	2.1 ± 0.7^{b}	0.4 ± 0.3	8.7 ± 2.4	1.3 ± 0.2	0.8 ± 0.2	
Sulfate only	1.2 ± 0.2	1.1 ± 0.2^{bc}	1.1 ± 0.3^{bc}	0.8 ± 0.2^{a}	0.3 ± 0.3	8.9 ± 1.7	1.1 ± 0.2	0.7 ± 0.0	
Acetate–molybdate	1.9 ± 0.7	0.5 ± 0.6^{a}	0.5 ± 0.6^{c}	0.1 ± 0.2^{d}	0.2 ± 0.4	8.7 ± 0.8	1.3 ± 0.4	1.2 ± 0.5	
Butyrate–molybdate	1.2 ± 0.4	0.9 ± 0.2^{ab}	1.0 ± 0.3^{bc}	b.d.	0.3 ± 0.2	7.1 ± 1.8	1.6 ± 0.3	0.9 ± 0.1	

Different letters (a, b, c, d) indicate significant differences (as determined by ANOVA analysis) between treatments ($P = 0.05$) for those PLFA which were microcosm-differentiating in the PCA.
b.d., below detection.

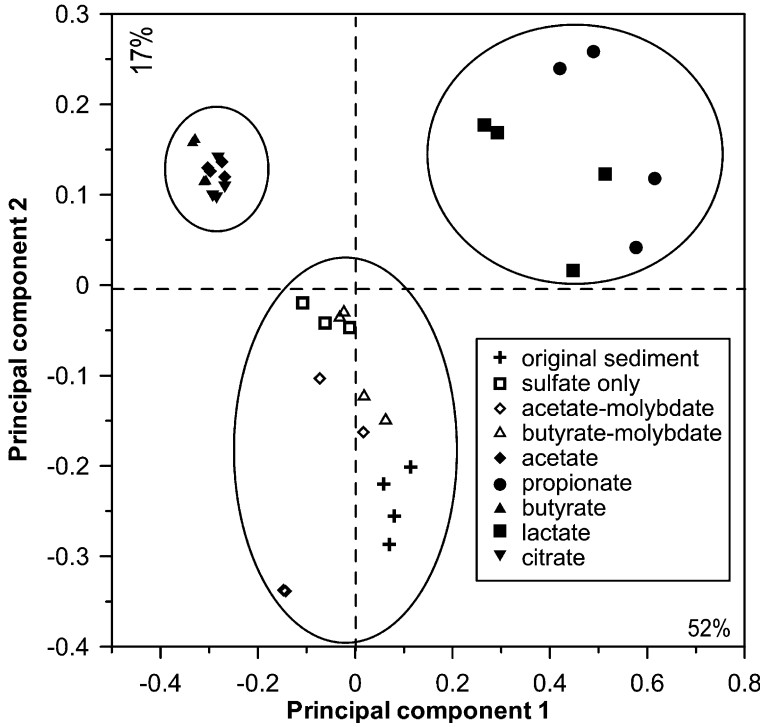

Fig. 1. Principal component analysis with 17 PLFA commonly found in SRB (Table 3). Each treatment is represented by four data points (two in each of the duplicate microcosms).

likely to be abundant in acetate, butyrate and citrate treatments and 17:1ω6c, 15:1ω6c, 15:0, and possibly 17:1ω8c were dominant in microcosms amended with lactate and propionate. All other PLFA did not contribute substantially to the variability of PLFA profiles in microcosms. A closer look at the relative amounts of the mentioned PLFA in the microcosms (Table 3) reaffirms the PCA results. Significantly higher percentages ($P = 0.05$) of 17:1ω6c, 15:1ω6c, and 15:0 occurred in microcosms amended with lactate or propionate as compared to the

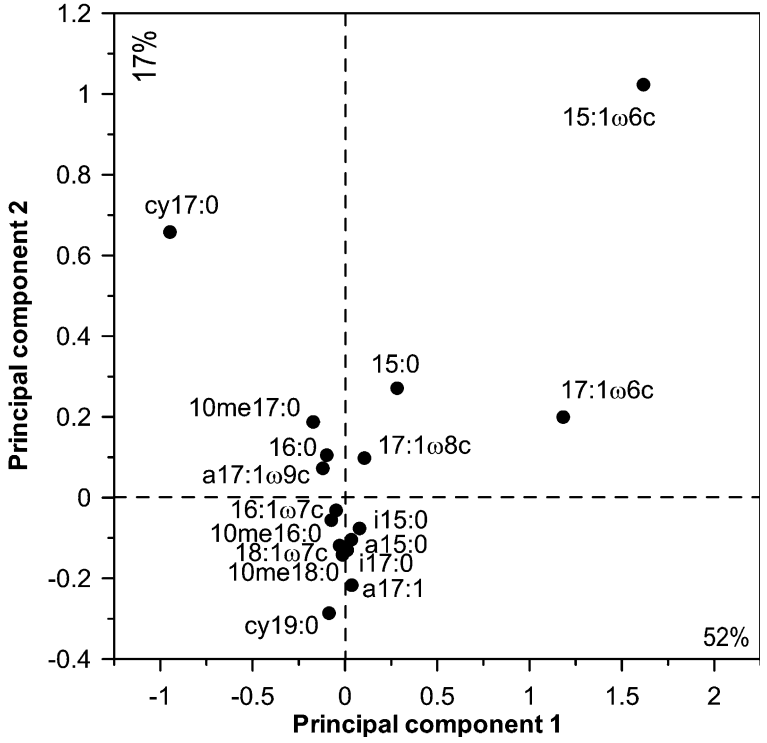

Fig. 2. Factorial map of 17 SRB-related PLFA in the principal component analysis presented in Fig. 1.

other microcosm series (as indicated by different superscript letters in Table 3). In contrast, cy17:0 exhibited significantly higher percentages in acetate, butyrate, and citrate as compared to all other treatments. The PLFA i17:1ω7c, commonly found in *Desulfovibrio* [21], was not detected in any of the microcosms.

4. Discussion

4.1. Sulfate and carbon source consumption

Sulfate reduction in the original sediment was more limited by the availability of sulfate than by that of organic carbon sources since substantial sulfate reduction occurred in microcosms with no extra carbon source amendment (Table 1). This concurs with the general concept that microbial activity in contaminated environments is typically electron acceptor-limited [47].

Molybdate completely inhibited sulfate reduction in our control microcosms. Consequently, lower total acetate and butyrate mineralization in molybdate treatments as compared to treatments without molybdate suggests that SRB were the major group responsible for the turnover of these carbon sources. This result is supported by FISH results (probes SRB385, SRB385-Db) showing that SRB were dominant members of the active bacterial community in the aquifer microcosms.

But FISH results also indicated the presence of *Archaea* in most treatments (probe Arch915, Table 2), which explains the methanogenic activity observed in all but the autoclaved microcosms. Considerable acetate consumption in acetate–molybdate treatments further indicated the presence of methanogens, since acetate could not have been mineralized by SRB in these treatments.

On the other hand, a comparison of carbon source turnover between molybdate treatments and respective treatments without molybdate indicates that the majority of sulfate reduction observed in the latter was coupled to the mineralization of amended rather than endogenous carbon sources. For example, considerably less acetate was mineralized in molybdate treatments than in treatments without molybdate (0.55 versus 1.38 mmol on average, Table 1). Hence, we may assume that on average at least 0.83 mmol of acetate was mineralized by SRB in treatments without molybdate. But mineralization of acetate by SRB is coupled to the reduction of an equal amount of sulfate (Eq. 5). Thus, at least ~70% of observed sulfate consumption in acetate-amended microcosms was coupled to acetate mineralization. Following the same line of reasoning, almost all of the observed sulfate consumption in butyrate-amended microcosms was coupled to butyrate mineralization. We would expect a similar behavior in propionate, lactate, and citrate microcosms, as these carbon sources are also easily degradable. Nevertheless, since we did not perform molybdate controls for propionate, lactate and citrate, we cannot unequivocally assess the contribution of amended carbon source mineralization to overall sulfate consumption in these experiments. Likewise, the contribution of sulfate reduction to amended carbon source mineralization remains uncertain in these experiments. Hence, observed stoichiometric ratios (Table 1) for propionate, lactate, and citrate microcosms have to be interpreted with caution.

4.2. Community structure

Principal component analysis of PLFA profiles and FISH results indicated that no detectable changes of the SRB community were induced in control treatments as compared to the original sediment. In contrast to PLFA results, FISH data showed that the overall activity of SRB (probes SRB385-Db and SRB385) was enhanced in the control treatment with sulfate but no extra carbon source amendment (Table 2). Conversely, using PCA, carbon source-amended microcosms were separated into two groups that diverged from the original sediment (Fig. 1). Hence, selective stimulation of SRB upon carbon source addition occurred. Note that carbon source additions over the incubation period accounted for less than 5% of total carbon already present in the microcosms, i.e. sediment-borne organic matter and PHC. Consequently, carbon source additions represented a minor stimulation of the bacterial community in the microcosms. The PCA data suggested that at least two different sulfate-reducing key-players occurred in the microcosms.

The presence of 17:1ω6c in the original sediment suggested members of the genus *Desulfobulbus* [3,48], however, 15:1ω6c, a PLFA frequently found in this genus [48], was lacking. Nevertheless, 15:1ω6c appeared in propionate- and lactate-amended microcosms. In the PCA (Fig. 1), this compound formed, together with 17:1ω6c, 15:0 and 17:1ω8c, a cluster of PLFA characteristic for the genus *Desulfobulbus* [3,20,48], revealing that *Desulfobulbus* proliferated upon lactate and propionate addition. This result was also corroborated by FISH results (probe 660, Table 2). *Desulfobulbus* is an incomplete oxidizer of lactate and propionate [6], and our stoichiometric data (Table 1) agree with incomplete oxidation of these carbon sources. Hence, our results are in agreement with those from other researchers who also found stimulation of *Desulfobulbus* upon lactate and propionate additions to estuarine and marine sediments using PLFA analysis or 16S rRNA-targeted oligonucleotide probes [27,48,49].

The biomarker for *Desulfovibrio*, i17:1ω7c [3,24], was not found in the original sediment nor in any of the microcosms, and FISH analysis (probes DSV698, DSV1292, DSD131, DSV214, and DSV407) showed that specific members within this genus were not detected in any microcosms at our detection limit. This seems contradictory to the fact that in theory the difference between counts with probe SRB385 and probe 660 should be *Desulfovibrio*.

However, care has to be taken since SRB385 also detects a range of other anaerobic bacteria [4]. The five *Desulfovibrio* probes we used encompass 84% of all *Desulfovibrio* sequences in the database [4]. Hence, if there had been *Desulfovibrio* in our microcosms they were either unaccounted for by the probes or they are unknown *Desulfovibrio* species, or indeed *Desulfovibrio* were not present and probe SRB385 detected non-target species. These results are surprising because *Desulfovibrio* was detected in water samples collected from the same aquifer but from a different well [50]. Spatial heterogeneity of bacterial community composition and/or activities or differences between attached and suspended bacteria may be a cause for this discrepancy. Nevertheless, several studies showed contradictory results concerning the importance of *Desulfovibrio* spp. at different sulfate-reducing marine and freshwater sites [48,49,51–53]. We suggest that *Desulfobulbus* spp. were the active lactate degraders instead of *Desulfovibrio* in our lactate-amended microcosms. Alternatively, *Desulfobulbus* may have mineralized propionate derived from fermentation of lactate [49] in these microcosms.

Although cell counts were low, indicating low bacterial activities, approximately 25% of the total active *Bacteria* (probe EUB338) belonged to the *Desulfobacteriaceae* (probe SRB385-Db) in the original sediment. Although the presence of 10me16:0 in the original sediment may be indicative for members of the genus *Desulfobacter* [23–25], cy17:0, which was also considered a specific biomarker for this genus [23,25], was lacking. This PLFA appeared in acetate-, butyrate- and citrate-amended microcosms after incubation and in the PCA, cy17:0 formed, together with 10me17:0, a second group of PLFA (Fig. 2) characteristic for *Desulfobacter*-like bacteria [3,23,25,54]. Hence, *Desulfobacter*-like bacteria seemed to have proliferated upon acetate, butyrate and citrate additions. *Desulfobacter* spp. are specialized on acetate [6] and probably mineralized acetate derived from fermentation of butyrate and citrate in the respective microcosms. However, as opposed to PLFA data, detection of *Desulfobacter* spp. using FISH was $<1\%$ in all treatments. Considering these data, we suggest that either *Desulfobacter* spp. were present but not active enough to show a strong fluorescence signal, or that an unknown, *Desulfobacter*-related SRB, which cannot be detected using probe DSB985, was responsible for acetate degradation. At this point we can rule out members of the genus *Desulforhabdus* as acetate-degrading bacteria because this genus is known to possess cy17:0 but also 17:1ω6 in the same range [3] and the latter PLFA was not detected in acetate, butyrate or citrate treatments. Furthermore, bacteria belonging to the genus *Desulfobacterium* were probably also not responsible for acetate degradation in microcosms since they do not contain cy17:0 [24]. In addition, *Desulfotomaculum*, a Gram-positive acetate degrader [28,55], was not detected by FISH using the specific probe [42]. Several other authors found differing results concerning the stimulation of *Desulfobacter*-type bacteria in marine or freshwater sediment slurries upon acetate addition [3,27,48,49,56]. However, it is still unclear which conditions favor the dominance of *Desulfobacter*-type or other acetate-degrading SRB in the environment.

Numbers of *Archaea* were substantially lower in the sediment or in the microcosms presented here than in water samples collected from the same aquifer [50]. Again the cause may be spatially different communities and/or activities or differences between attached and suspended populations.

4.3. Conclusions

Care has to be taken when interpreting the presence of specific PLFA in the environment as indication for the presence of a specific population [26,27]. However, the design of our aquifer microcosms minimized this problem as SRB were favored over other bacteria, and therefore changes in PLFA compositions most probably reflected changes in the SRB community. This study showed that SRB community changes in microcosms can be followed using statistically supported PLFA analysis in combination with FISH and metabolic data. Members of the genus *Desulfobulbus* were rather inactive in the original sediment but were stimulated upon propionate and lactate additions. The same is probably true for *Desulfobacter*-like bacteria, which were stimulated in acetate-amended microcosms. Conversely, we did not find evidence that *Desulfovibrio* was stimulated with lactate.

All carbon sources applied to the aquifer microcosms induced changes in the SRB community. Therefore, following the reasoning of Parkes et al. [48], these carbon sources may not necessarily be present or used by the SRB in situ in this sediment. Instead, SRB may be directly involved in degradation of PHC constituents [29]. We are aware of the problem that the dominant types of SRB in microcosms may change with increasing incubation time, especially on substrates that are incompletely oxidized [48]. However, the purpose of this study was not to investigate a population development time course. This could be the topic of future studies. Further information on SRB communities in contaminated aquifers may be obtained using DNA extraction and PCR-based approaches targeting specific SRB genes [57,58].

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